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Abstract: Individual carrying mutations in p53 or BRCA1 genes are predisposed to a variety of cancers, both tumor suppressor genes have been implicated in establishing genome stability by participating in DNA damage pathways. Inactivation or mutation of p53 is seen in variety of cancers including breast cancers. There have been discrete p53 mutations in African American (AA) cohort, which were different White American (WA) cohorts. Mutations in BRCA1 gene accounts for about 50% of inherited breast cancer cases and 80% of families predisposed to breats and ovarian cancers. However, somatic mutations of BRCA1 gene are absent in sporadic cancers. Recent studies have shown that inactivation of BRCA1 occurs via the hypermethylation of the promoter region of the BRCA1 gene. The results of this study show that: 1) the number of p53 negative cases were lower in AAs as compared to WAs, 2) a markedly p53 overexpression was observed in AAs; and also the number of p53 mutations were more in AAs as compared to WAs, 3) hypermethylation of promoter of BRCA1 gene was seen in cases where p53 was muted. This study, when complete will establish a causal variation in AAs as compared to WAs.

## INTRODUCTION

Breast Cancer (BC) is the most common cancer in women indeveloped countries and is also a leading cause of death among women. The main objective of our research is two fold; 1) To identify alterations in genes that are involved in tumor development, progression and metastasis. In this study we the analyse the mutations of the tumor suppressor gene p53 are among the most common genetic defects in cancer cells, and in several studies alterations in p53 in breast cancer have been associated with a poor prognosis. Studies have suggested that African American (AA) have a different spectrum of p53 mutations compared to White American (WA) although the specific mutational differences between these studies are somewhat conflicting (Blaszyk et al., 1994; Shiao et al., 1995). Also, AAs with p53 mutations had a much poorer prognosis compared to WAs. We propose to study the mutational spectrum of p53 gene in AW and AA.
Germline alterations in BRCA1 gene is responsible for 50% of familial breast cancer (Freidman et al., 1994; Futeal et al.,1994). BRCA1 gene is not shown to be mutated in sporadic breast cancer (Marajver et al., 1995; Berchuck et al.,1998). However, recent studies have demonstrated that inactivation of BRCA1 gene in sporadic cancer can occur due to hypermethylation of promoter region of BRCA1 gene (Esteller et al., 2000; Hedenfalk et al., 2001), and this may explain a mechanism by which BRCA1 gene get inactivated in some sporadic cancers. Furthermore, loss of BRCA1, a tumor suppressor gene in sporadic form of breast cancer may offer a novel mechanism of its role in tumor initiation.

Despite this fact, there have been no published studies determining whether breast cancers arising in AA-Women have an increased frequency of defects in both the p53 and BRCA1 genes, and whether there are specific types of defects associated with these two genes that distinguish these tumor groups. Also, mutations in p53 and BRCA1 have been studied; however, the possible relationship of p53 and BRCA1 hypermethylation has not been defined.

### Hypothesis/Rationale/Purpose

While overexpression of p53 protein occurs with similar frequency in breast cancers from Caucasian and AA-Women, limited published data suggest that tumors from the latter group exhibit a different spectrum of p53 mutations (Blaszyk et al., 1994; Shiao et al., 1995). Therefore, we assessed sporadic breast cancers from AA and AW for the following:

1) Overexpression of p53 protein and/or sequestration of p53 protein in the cytoplasm by immunohistochemical (IHC) techniques using p53-specific antibodies.
2) Mutational analysis of p53 gene, employing standard molecular biological techniques using DNA extracted from microdissected tumor tissues.
3) Global and specific gene expression
4) Overexpression of the proto-oncogene CpG islands of BRCA1 promoter in chemically modified DNA and then using methylation-specific PCR

Thus, in this project we tested the hypothesis that in breast cancers from AA women, the

frequency of p53 defects in individual tumors from AA women is higher than that observed in White women, and that the changes in p53 are associated with hypermethylation of BRCA1.

**Methods**

Tissue blocks were obtained thus far for 94 women who have been diagnosed with breast cancer at Crozer-Chester Medical Center located near Philadelphia, PA and the Medical College of Pennsylvania Hahnemann University. In addition, we had obtained tumor samples from New-York Presbyterian Hospital in New York City.

*Immunohistochemical Analysis:* Formalin-fixed, paraffin-embedded archival surgical tissue blocks were identified that corresponded to the appropriate patient charts. The pathology departments at MCP and Crozer identified the tissue blocks by slide review, and this along with the pathology chart was forwarded to the American Health Foundation. Deparaffinized and rehydrated sections were retrieved in a citrate buffered solution via heat-induced epitope retrieval for 6 min in a microwave. Endogenous peroxidase activity will be blocked by incubating with 3% hydrogen peroxide for 15 min. Nonspecific protein binding is blocked using serum free protein block (DAKO) for 7 min. A standard avidin-biotin complex procedure is used (ABC, Vector Labs). For identification of tumors showing over-expression of p53 protein, the mouse monoclonal antibody PAb-1801 (Lab Vision) was employed and graded modified from the method of Elledge et al., (1994) using a scale of 0-6, in which positivity is 3. In this scale, intensity was graded as 0-3 and proportion of positive cells 0 < 10%, 1 = 10-32%, 2 = 33-66%, 3 > 66%.

***DNA Extraction from Formalin Fixed Tissue:*** Genomic DNA was extracted from paraffin fixed breast tissue sections following paraffin removal procedure using xylene-ethanol using standard procedures (Shambrook and Russell, 2001). Aliquots of the purified supernatant were taken directly out of the sample extraction tube and used for PEP reactions; the remaining sample was stored at -20°C.

***PCR-SSCP analysis and direct DNA sequencing for p53 mutations:*** The genomic DNA (50-150 ng) was be amplified by PCR using oligonucleotide primers designed for TP53 gene from published sequences (Orita et al., 1989; Jhanwar-Uniyal and Gulati, 1998). PCR-SSCP analysis of Exons 4, 5,6,7,8 and 9 of the p53 gene, with nucleotide length of 139 bp to 330 bp, was performed using a published technique (51). These regions contained domains of p53 highly conserved among species, and they are also site of frequent mutations in breast cancer. Briefly, 50 ng of genomic DNA will be amplified with 0.4 umol/L of forward and reverse primers, dNTPs (2.5 umole/L), 10 mM Tris (pH 8.8)-$MgCl_2$ buffer and 0.2 units of *Taq* polymerase (Perkin Elmer-Cetus, USA) in a final volume of 25 :l. Conditions for PCR will be used as described (Orita et al., 1989; Jhanwar-Uniyal and Gulati, 1998) and amplification was being carried out in an automated DNA Thermal Cycler (Eppendorf). An aliquot of each was diluted with 0.1 sodium docecyl sulfate (SDS) and 10 mmole/L EDTA, and further diluted with 1:1 with sequencing stop solution (95% formamide, 20 mmole/L EDTA. 0.05% bromophenol blue, and 0.05% xylene cyanol FF). Samples were heated at 95□C for 5 min and, chilled on ice, and immediately loaded onto a 6% polyacrylamide (acrylamide: N, N'-bisacrylamide =49:1) in Tris/EDTA/borate buffer, stained with ethedium bromide and photographed.

**Methylation of BRCA1 gene:**

**Modification of genomic DNA:**
Bisulfide modification of genomic DNA was performed in order to chemically modify cytosine nucleotide to uracil (Esteller et al., 2000). In the reaction, all cytosines are converted to uracil, but those that are methylated (5-methylcytosine) are resistant to this modification and remain as cytosine. This altered DNA can then be amplified and sequence and provides detail information of the region of the methylation of CpG sties for a specific genes. DNA (1 ug) was denatured using NaOH for 10 min at $37^0$C followed by bisulfide treatment and an incubation for 16 hrs at $50^0$C. After which DNA was purified. Followed by a treatment with NaOH and then ethanol precipitation.

**BRCA1 promoter methylation:**
We have used promoter specific to BRCA1 gene and specifically design for the methylated and unmethylated sequence. Modified DNA was amplified with BRCA1 promoter specific primers. PCR conditions were as follows: $96^0$C for 5 min. then 35 cycle of $96^0$C for 20 sec., $60^0$C for 20 sec, $72^0$C for 90 sec; and finally 5 min at $72^0$C. Controls without DNA were performed for each set of PCRs. Amplified PCR product was electrophorised, stained with ethidium bromide and directly visualized under UV illumination.

**Results**

**Study population:**
We have thus far obtained breast carcinoma samples 34 AA women and 60 WA women from the two hospitals in Pennsylvania, namely Crozer and MCP. Table I represents number and age of these cohorts.

| | **Race** | | **Age at Diagnosis** | |
|---|---|---|---|---|
| | **African American** | **White American** | **African American** | **White American** |
| MCP | 30 | 11 | 30-91 | 43-91 |
| Crozer | 4 | 49 | 51-74 | 36-75 |
| Total | **34** | **60** | **30-91** | **36-91** |

**Table 2: Distribution of p53 Scores:**

| **P53 Grading** | **0** | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|---|
| **African American (AA)** | 7 | 10 | 5 | 2 | 3 | 6 | 1 |
| **White American (AW)** | 22 | 16 | 5 | 6 | 3 | 1 | 4 |

- Only n=57 samples were analyzed for AW

**Table 3: Expression of Mutant p53 in African American and White Americans: Positive and Negative scores of P53:**

| p53 Negative (Score 0-3) | | p53 Positive (Score 4-6) | |
|---|---|---|---|
| AA | AW | AA | AW |
| 24 (70%) | 49 (86%) | 10 (29%) | 8 (14%) |

*Only n=57 samples were analyzed for AW

Accumulation of mutant P53 protein resulted in a higher immunohistochemical signal to the cell of malignant breast epithelium and data are presented in Table 2 and 3. p53 positivity which confers a poor prognosis, is notably more prevalent in AW as compared to AA. Of all samples analyzed 29% samples of AA origin display p53 positive staining, while only 14% of AW displayed high score of p53. Moreover, 86% of AW showed low p53 staining while 70% of AA showed low staining. It is interesting to note that 0 score was seen in 20% of all AW cases examined. However, 39% of AW breast samples display 0 scores. Usually, a strong correlation between p53 positive staining is associated with tumor proliferative rate.

**p53 gene mutation analysis:** Mutational analysis of p53 gene was done in samples which displayed high scores in P53 immunostaining (Scores 4-6). Some samples showing p53 negative staining *and had* ER and PR negative, were also studied for p53 mutation. PCR-SSCP analysis of these samples demonstrated a strong link between high scores in P53 immunohistochemical analysis and p53 mutation. Although, so far we have done p53 gene analysis only Exon 5-9, also known as hot-spot zone of the gene. Our study show that samples from 2 AA subjects, which display a high score of p53 immonohistochemistry, had mutations of p53 in Exon 7. Both these subjects had infiltrating ductal carcinoma invasive. One of these two subjects was diagnosed with breast cancer at relatively young age of 30 and had negative ER and PR. Status. One other sample from AA subject with medullary carcinoma displayed high p53 IHC and showed a mutation of p53 gene in Exon 5. Our study demonstrated that 30% of samples, displaying high IHC for p53 protein, from AA origin, show mutation in p53 gene (Exon 5-9 studied so far). We detected no mutation in p53, Exon 5-9 in six samples which displayed high p53 IHC. However, We detected two p53 mutation in AW sample. In addition, we show a p53 mutation in Exon 5 from DNA derived from AW, who show a border line a ER and PR, and had high proliferative index.

We are in process of standardizing the techniques to determine the p53 mutation in Exons 2-4 and 10-11 and we are planning to perform mutational analysis in breast cancer samples which display high scores for p53 levels using IHC and fail to show mutation in hot-spots zone (Exon 5-

9), examined in our current analysis.

**Methylation of BRCA1 Promoter:**

Our results, thusfar show hypermethylation of BRCA1 promoter in samples which display p53 mutation. Three samples, from AA populations had high p53 expression and two of these three had p53 mutation in Exon 7 (one is still being studied for Exons, 2-4,and 10,11). It is interesting to note that 2 of these subjects had onset of breast cancer at a relatively very young age (age 30 and 33 years). We had also observed hypermethylation of BRCA1 in sample from AW; two of them show ER and PR negative status. This is a preliminary study. We are in process of performing such a study in other samples to establish a strong correlation. This epigenetic mechanism of gene inactivation of BRCA1 gene is observed in sporadic cancer and may explain an inactivation of tumor suppressor gene besides mutation of loss of heterozygosity (LOH).

**Discussion:**

The project has been complicated but is progressing well. We have confirmed histopathological diagnosis in H&E stained sections from the tumor blocks. Immunohistochemical determination of p53 is completed and graded. Mutation of p53 gene with respect to Exon 5 through 9 has been done. Mutational analysis of other Exons (2-4 and 10 and 11) of p53 gene is currently underway. To-date, we have some data to suggest a racial disparately exists in-terms of genetic factors which may contribute to breast cancer. We wish to continue this work till it is ready for publication.

## KEY RESEARCH ACCOMPLISHMENTS

- P53 overexpression in our sample is greater in AA compared to WA women.
- The number of p53 mutations was greater in the AA compared to the WA women.
- An association between mutation in p53 gene and hypermethylation of the BRAC1 promoter.

## REPORTABLE OUTCOMES

Due to the recent completion of our results and since we plan to extend this study further, we have not reported any of the outcomes of this study at this time.

## CONCLUSIONS

We wish to request DOD grants us no-cost further extension in this grant. We feel this study is very important, and results of this report can decipher genetics variations elating to breast cancer. Furthermore, the finding of this study can help design unique diagnostic and treatment strategies. The identification of altered BRCA1, with p53 abnormalities, which are present in excess in primary breast cancer from AA women, provides a rationale for a future prospective clinical study.

This will be designed with sufficient statistical power to determine whether the loss of BRCA1 protein, with or without altered p53, is an independent prognostic biomarker. In the long-term, these studies may provide a novel means of developing targeted therapeutic agents.

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**Appendices:**

1) In our study, the number of African American (AA) subjects is significantly less (n=34) than American Whites (n=60). The main goal of this study is determine the genetic markers in AA which are responsible for aggressive kind of breast cancer in these population. To encorporate more AA subjects in this study, during past 6 months, we have established a close working relationship with Dr. William Thelmo, Chairman of Pathology, Wykoff Methodist Hospital, Brooklyn, New York. We have applied and received an IRB approval to conduct p53 gene mutation and BRCA1 inactivation studies in African American population.

2) Based on our findings, we have submitted an abstract to Annual Meeting of American Association for Cancer Research (AACR;2003). (Enclosed).

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## Involvement of p53 and BRCA1 genes in Breast Cancer in African-American and White Women

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The p53 and BRCA1 tumor suppressor genes play a key role in establishing genome stability. Inactivation or mutation of p53 is seen in variety of cancers including breast cancers. There have been discrete p53 mutations in tumors from African-Americans (AA) that were different from those observed in White Americans (WA). Germline mutations in BRCA1 have been reported in hereditary breast cancer, but somatic mutations of BRCA1 gene are absent in sporadic cancers. Recent studies have shown that inactivation of BRCA1 occurs via the hypermethylation of the promoter region of the BRCA1 gene. The purpose of this study was to clarify the role of two susceptibility genes as determinants or potential modifiers of outcome differences in African-American and White women diagnosed with breast cancer. AA breast cancer patients more frequently have clinical and pathological features of advanced disease and reduced survival than their white counterparts. Well-characterized tumor tissues from 94 breast cancer patients (34 AA and 60 WA) diagnosed at two Philadelphia hospitals were screened for mutations of p53 and BRCA1 inactivation using various methods including immunohistochemistry, DNA-modification followed by methylation-specific PCR, PCR-SSCP analyses and a direct DNA sequencing. Our results show that: 1) the number of p53 negative cases were lower in AAs as compared to WAs, 2) p53 overexpression of mutant protein was more commonly observed in AAs than WAs, and also the number of p53 mutations were greater in AAs compared to WAs, 3) hypermethylation of promoter region of BRCA1 gene occurred in cases where p53 was mutated with concurrent negative estrogen receptor (ER)/progesterone receptor (PR) status in both the AA and WA tumors. No BRCA1 inactivation was detected among the patients without p53 mutations or in those with p53 mutations and ER/PR positive tumors. We hypothesize that loss of expression of ER and PR proteins leads to genomic instability that may result from the inactivation of p53 via mutation and BRCA1 via hypermethylation. Data from this study suggest that variation in these genetic susceptibility factors may have prognostic significance in AA and WA breast cancer patients.
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